The Traditional Toxicologic Paradigm is Correct: Dose Influences Mechanism

Jay I. Goodman

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan

Dose influences mechanism; and over a wide range of doses, one can envision that mechanism will change with changing dose. This basic concept in toxicology is juxtaposed with the biologic importance of maintaining normal DNA methylation status to provide the focus of this paper. The idea that altered DNA methylation plays a variety of roles in carcinogenesis is compatible with three key features of this multistage process: clonal selection of abnormal cells in a progressive fashion, the reversibility of tumor promotion, and the multiplicity of tumor phenotypes. A relatively low capacity to maintain normal methylation status appears to explain, in part, the high propensity of the B6C3F1 mouse to develop liver tumors. This observation supports the view that a mouse liver tumor response is not an appropriate end point for human risk assessment. Additionally, it is suggested that altered DNA methylation can be viewed as a secondary mechanism underlying carcinogenesis. The knowledge that a chemical is acting by a mode of action involving a secondary mechanism can be used to support a safety factor or multiplicity of exposure approach to risk assessment. — *Environ Health Perspect* 106(Suppl 1):285–288 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-1/285-288goodman/abstract.html*

Key words: B6C3F1 mouse, carcinogenesis, DNA methylation, epigenetic, genotoxic, hypomethylation, mouse liver tumor, nongenotoxic, promotion, risk assessment, secondary mechanism

Introduction

In the 16th century Paracelsus noted: "What is there that is not a poison? All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison." There is a consensus that he was the first person to deal with dose in a quantitative manner and that he was, in general, discussing a threshold or no-effect level (1). This view is supported by clear historical examples regarding cancer and noncancer end points, e.g., chloroform-induced hepatocarcinogenesis in mice occurs after repeated oral administration only if individual doses are large enough to produce liver necrosis (2,3). Thus, it appears that Paracelsus' thinking is fundamentally correct. This paper will focus on this concept and its importance with regard to utilizing mode of action information to take a rational approach towards carcinogen risk assessment.

The Carcinogen Bioassay

The carcinogen bioassay is a qualitative test (4). However, our purpose is not to simply identify chemicals that can be labeled as carcinogens. On the contrary, the overall goal is to provide a reasonable estimate of the possible hazard that a chemical might pose to people under realistic conditions of exposure. Three key issues center around dose selection, dose–response relationships, and species-to-species extrapolation (5,6). Therefore, the bioassay should be approached more like a research project than simply a test because a rational approach to risk assessment requires the use of biologic information (7).

Dose Influences Mechanism

Dose influences mechanism; and over a wide range of doses, one can envision that mechanism will change with changing dose. Thus, a carcinogenic effect observed at a high dose is not necessarily expected to occur at lower doses (5), especially when dealing with nongenotoxic chemicals (8). For example, a report on the relationship between use of the maximum tolerated dose (MTD) and study sensitivity for detecting rodent carcinogenicity concluded:

> ...[an] important limitation of our analysis is that the range of doses used in NCI/NTP [National Cancer Institute/U.S. National Toxicology Program] long-term rodent studies is generally rather narrow, typically extending from 1/4 MTD to MTD or from 1/2 MTD to MTD. Thus, it could be argued that carcinogenic effects that are present at even the "lowest" of these doses are due to the same cell killing and compensatory mitogenesis effects that occur at the MTD (9).

Increased cellular proliferation may facilitate carcinogenesis because mitogenesis can facilitate mutagenesis (10). In general, there is a positive association between increased cell proliferation and carcinogenesis (11,12). However, in view of the complexities involved in the transformation of a normal cell into a frank malignancy it is not surprising that a one-to-one relationship between cell proliferation and carcinogenesis is not always apparent (13).

Dose Selection

A consideration of dose selection for the bioassay entails two primary questions: *a*) what is an appropriate range of doses, including the MTD, to use for chronic exposure; and *b*) after a bioassay has been completed, what are the appropriate doses to use to estimate the possible effect(s) the agent in question may produce in humans under realistic conditions of exposure? Two key principles underlie dose selection: *a*) it is not correct to make an assumption a priori that these doses are the same (i.e., a dose that appeared to be a reasonable MTD based on a 90-day study may turn out to be too toxic in a chronic study); and *b*) any high dose, no matter how high, that permits the test animals to survive long enough to develop cancer is not necessarily an appropriate dose to use for the purpose of a risk assessment (5).

Genotoxic versus Nongenotoxic Carcinogens

Genotoxic carcinogens are capable of interacting directly or interacting with DNA after being metabolized (14). Presumably,

mutagenesis provides a basis for their action. Nongenotoxic compounds appear to produce cancer either in a species-and/or dose-specific fashion (15), whereas most of the transspecies carcinogens are genotoxic, i.e., mutagens (16). However, many noncarcinogens are mutagens: 30% of 83 noncarcinogens were reported to be mutagens (17). Furthermore, only 30% of mouse liver-specific carcinogens are mutagens (17). If mouse liver tumors can be induced by nonmutagens it is logical to consider that such specific tumor responses by mutagens may occur independent of genotoxicity (18). Carcinogenesis involves a variety of alterations to the genome, including point mutations, chromosome deletions, and epigenetic phenomena (19). It is a mistake to equate mutagenesis with carcinogenesis (11).

The Role of Altered DNA Methylation in Carcinogenesis

We have made progress in discerning mechanisms involved in carcinogenesis by focusing on the variety of roles that alterations in DNA methylation (5-methylcytosine content of DNA) may play (20) and by placing an emphasis on testing the hypothesis that hypomethylation is an epigenetic, nongenotoxic mechanism involved in tumor promotion (21–23). Hypomethylation may occur by one or a combination of the following:

* a passive mechanism involving a failure to maintain the normal symmetrical pattern of methylation due to a decreased ability to carry out the *S*-adenosylmethionine-requiring maintenance methylase reaction (24,25), either during periods of cell replication [reviewed in Razin and Cedar (26)] or as a consequence of carcinogen adducts in DNA (27–29); and
* an active mechanism involving the removal of 5-methylcytosine from DNA and its replacement with cytosine (30).

In this context, it should be noted that the U.S. Environmental Protection Agency's proposed guidelines for carcinogen risk assessment state:

A failure to detect DNA damage and mutation induction in several test systems suggests that a carcinogenic agent may act by another mode of action....It is possible for an agent to alter gene expression...by means not involving mutations....For example, perturbation of DNA methylation patterns may cause effects that contribute to carcinogenesis (31).

The idea that altered DNA methylation plays a variety of roles in carcinogenesis, none of which are mutually exclusive, and involves genetic and epigenetic events (20,32), is compatible with the view that carcinogenesis is a multistep/multistage process that exhibits three key features:
* There is a clonal evolution of tumor cell populations involving a stepwise selection of sublines that are increasingly abnormal both genetically and biologically and have a selective growth advantage over adjacent normal cells and most of the variants are eliminated (33).
* The promotion stage is reversible in the operational sense (34).
* Tumors exhibit a multiplicity of phenotypes—even those tumors arising in a particular organ spontaneously or after specific carcinogen exposure (35).

Altered DNA methylation may result in an altered pattern of gene expression that could provide subpopulations of cells with a growth advantage (36–40). The phenomenon of *de novo* methylation provides the potential for reversal of hypomethylation [reviewed in Counts and Goodman (20,22,37)] and altered DNA methylation may result in an altered pattern of gene expression that offers the potential for multiple tumor phenotypes.

Tumorigenesis in mouse liver is used as our model system (36,37,39–41) and phenobarbital serves as a nongenotoxic rodent tumor promoter (42). We use the B6C3F1 (C57BL/6 × C3H/He) mouse, which is highly sensitive to the development of spontaneous and chemical-induced liver tumors (43,44). This may be explained by the inheritance of hepatocarcinogen sensitivity genes from the paternal C3H/He that influence the promotion stage of carcinogenesis (45,46,47). Our experimental approach permits relevant comparisons to be made between the B6C3F1 mouse and its paternal C3H/He strain that is also highly sensitive and the relatively resistant maternal C57BL/6 strain (43,44). The results of our research indicate that the B6C3F1 mouse is deficient with regard to is ability to maintain normal DNA methylation. This appears to underlie in part its uniquely high susceptibility toward development of liver tumors (22,23,36,39,40). We believe that hypomethylation is relevant to tumorigenesis in both rodents and humans (23) and that humans may be less susceptible than rodents in part because of a better ability of human cells to maintain normal patterns of DNA methylation [reviewed in Counts and Goodman (23) and Counts et al. (39)].

The Role of Mouse Liver Tumors in Risk Assessment

Fourteen years ago an advisory committee examined the role of mouse liver tumors in risk assessment and concluded "...it would be prudent to severely limit or even eliminate exposure to potentially genotoxic chemicals which induce malignant tumors at multiple sites or in multiple species, and at low exposure levels. Less concern is warranted in the case of chemical induction of tumors only in mouse liver...." (48). Data accumulated in recent years are supportive of this position and justify its expansion. It is not appropriate to make human risk assessment decisions based on a mouse liver tumor response. However, in those situations where the results of a bioassay indicate that the mouse liver is one of several sites where an increased tumor incidence occurs, the mouse liver tumor response and other target sites for tumorigenesis should be evaluated with regard to the mode of action of the chemical in question. Safety assessment for those chemicals (especially nongenotoxic chemicals) acting through a threshold-exhibiting mode of action should be based on a safety factor or multiplicity of exposure approach (23,39). A constructive conceptual framework that provides guidance for the use of mode of action data for carcinogens in the regulation and classification of carcinogens has been presented (8).

Altered DNA Methylation as a Secondary Mechanism in Carcinogenesis

The maintenance of nascent DNA methylation status should be regarded as a fundamental homeostatic mechanism. Accordingly, it is appropriate to invoke the secondary mechanism concept (49) in this context. Altered DNA methylation can be viewed as a secondary mechanism involved in carcinogenesis; assessment of this parameter may provide insight leading to a more rational interpretation of animal studies for human risk assessment (23). Therefore, an examination of DNA methylation status should be considered for inclusion as an ancillary component (e.g., in addition to standard approaches involving histopathology, assessments of cell proliferation *in vivo*, and *in vitro* tests for genotoxic potential) of both subchronic studies and the carcinogen bioassay (22,23,39). This can aid in discerning the mechanism of action (e.g., a possible nongenotoxic, threshold-exhibiting mechanism) of the chemical being evaluated. In

addition, use of this information in conjunction with standard approaches could facilitate a rational approach to dose setting and the selection of appropriate doses for risk assessment, e.g., if toxicity occurs only at doses above those that cause altered DNA methylation in the target organ(s), these data could aid in providing the basis for placing a proper emphasis on lower doses (23,39). This suggested strategy is consistent with a recently proposed set of principles for the selection of doses in chronic rodent bioassays (50). However, it would not be appropriate to consider measurement of alterations in DNA methylation as a short-term test for carcinogens (22,23,39).

Conclusion

Emphasis should be placed on research that may discern probable thresholds for the carcinogenic effect of carcinogenic agents, especially nongenotoxic chemicals (5,23). This must involve hypothesis-driven research and must be based on insight regarding the mode of action of the chemical of interest (8). The practical significance here is that the proposed strategy can provide the basis for a safety factor or multiplicity of exposure approach to risk assessment for those chemicals for which a likely threshold can be demonstrated (5,51). This is a rational approach to risk assessment (Figure 1).
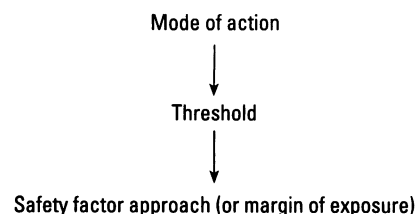
Mode of action

↓

Threshold

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Safety factor approach (or margin of exposure)

Figure 1. A rational approach to risk assessment. For those chemicals that act by a nonlinear, threshold-exhibiting mode of action, use of a safety factor or margin of exposure provides a rational approach to risk assessment. Under the circumstances described, a risk estimate based on a simple linear extrapolation from high dose to low dose is not appropriate.

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